Cellulases: biosynthesis and applications

DEWEY D. Y. RYU
The Korea Advanced Institute of Science, P.O. Box 150, Chung-Ryang-Ri, Seoul, Korea

MARY MANDELS
US Army Natick Research and Development Command, Natick, MA 01760, USA

Summary. *Strains of* Trichoderma, *particularly* T. reesei *and its mutants, are good sources of extracellular cellulase suitable for practical saccharification. They secrete a complete cellulase complex containing endo- and exo-glucanases plus β-glucosidase (cellobiase) which act syngergistically to degrade totally even highly resistant crystalline cellulose to soluble sugars. All strains investigated to date are inducible by cellulose, lactose, or sophorose, and all are repressible by glucose. Induction, synthesis and secretion of the β-glucanase enzymes appear to be closely associated. The composition and properties of the enzyme complex are similar regardless of the strain or inducing substrate although quantities of the enzyme secreted by the mutants are higher. Enzyme yields are proportional to initial cellulose concentration. Up to 15 filter paper cellulase units (20 mg of cellulase protein) per ml and productivities up to 80 cellulase units (130 mg cellulase protein) per litre per hour have been attained on 6% cellulose. The economics of glucose production are not yet competitive due to the low specific activity of cellulase (0.6 filter paper cellulase units/mg protein) and poor efficiency (about 15% of predicted sugar levels in 24 h hydrolyses of 10–25% substrate). As hydrolysis proceeds, the enzyme reaction slows due to increasing resistance of the residue, product inhibition, and enzyme inactivation. These problems are being attacked by use of pretreatments to increase the quantity of amorphous cellulose, addition of β-glucosidase to reduce cellobiose inhibition, and studies of means to overcome instability and increase efficiency of the cellulases. Indications are that carbon compounds derived from enzymatic hydrolysis of cellulose will be used as fermentation and chemical feedstocks as soon as the process economics are favourable for such application.*

Introduction

For a long-range solution to our resources problems of energy, chemicals, and food, cellulose is the only renewable carbon source that is available in large quantities. The amount of carbon fixed by photosynthesis using solar energy has been estimated at 100 billion tons per year and half of this as cellulose. The fraction which can be collected at a reasonable cost is low but could be increased significantly by better management. The production of biomass is probably one of the most efficient methods of harnessing solar energy.

Cellulose can be hydrolysed to soluble products by acids or enzymes. Processes for acid hydrolysis of cellulose exist and new technologies are being researched. Drawbacks of the acid process include degradation of the product, interaction of acid with non-cellulosic materials in crude cellulosics and corrosion of equipment resulting in low yields, impurities in the syrups, and high capital costs. In the long run an enzyme process should be preferable as has been the case for starch hydrolysis.

Successful utilization of cellulosic material as a renewable carbon source depends on the development of economically feasible process technologies for the production of cellulase, microbial and enzymatic hydrolysis of cellulosic material to low molecular weight products like hexoses and pentoses, and chemical and/or biological conversion of the hydrolysis products to other useful products to be used as liquid fuel, chemical feedstock, and food materials.

This review assesses briefly the economics of current cellulase process technology, describes the state-of-the-art of the cellulase technology, and suggests important areas for future research and development. Most of the discussion is limited to cellulase systems from *Trichoderma.* Interesting work on other cellulases[11] or direct conversion of cellulose by aerobic and anaerobic processes[209] has been instructive but these are still far from economic feasibility and are not reviewed.

The reference materials used for this paper are organized into five groups: (a) Recent issues of special symposia and proceedings volumes;[1–9] (b) a brief economic assessment of cellulase technology;[10–20] (c) production and biosynthesis of cellulase;[21–99] (d) enzymatic hydrolysis of cellulose;[100–181] (e) applications of hydrolysis products.[182–212]

Background and review

About 1950, Dr Elwyn Reese and his coworkers at the Natick Laboratories identified *Trichoderma* strains which produced an active and well-balanced cellulase complex. Many other strains having cellulase activity have been isolated and reported[11] but most do not produce adequate levels of extraceullar cellulase for practical use, i.e. extensive hydrolysis of high concentrations of cellulose. Practical saccharification data has been published for *Penicillium,*[56,211] *Sclerotium rolfsii,*[210] and *Thermomonospora*[212] cellulases but most studies have utilized cellulases from *Trichoderma viride*[157–159] or *Trichoderma reesei.*[101–103,107,114–116, 119, 124,130,131] Other *Trichoderma* species such as *T. lignorum* and *T. koningii* also appear to produce active cellulases. The mutant strains of *Trichoderma* in use including QM9123 and QM9414,[50] MCG77[32] and Rutgers C30 and NG14[57] are all descendants from QM6a, originally believed to be a *T. viride* but this strain is now recognized as a new species, *T. reesei.*[78]

0141–0229/80/020091–12 $02.00

The advantage of *Trichoderma* is that it produces a complete cellulase with all the components required for hydrolysis of crystalline cellulose, and this cellulase is resistant to chemical inhibitors[167] and stable in stirred tank reactors at pH 4.8, 50°C for 48 h or longer. The disadvantages are that *Trichoderma* does not digest lignin and the cellulases have a low specific activity, are product inhibited, contain only low levels of cellobiase and are slowly inactivated under reactor conditions at 50°C, and more rapidly inactivated at higher temperatures. Nevertheless, it is the best cellulase available today.

Cellulase process variables have been studied by many in an attempt to optimize the fermentation process variables and improve the cellulase productivity.[21,23,25,29,40,41,54,72,82,83,90,100]

Various substrates have been evaluated for cellulase production using agricultural residues,[30,31,56] soluble substrates,[77,78] whey,[48] a high level of cellulose,[62,79] hemicelluloses,[35,38,47] and other sources. The effect of ammonia assimilation,[80] amongst other factors, on cellulase production, has also been examined. Solid and semisolid cultures have both been used[86,88] and good cellulase resulted.

Serious effort for the separate and independent production of β-glucosidase has been noted.[81,84] Biosynthesis and excretion of cellulase have also been studied.[30,43] A few review articles on cellulase production have also appeared,[27,42,44] Some authors have employed continuous culture systems to study the physiological parameters,[70] growth kinetics,[28] transient response,[67] and cellulase productivity.[55,68,71]

Cellulase assays are complicated by the multiplicity of enzymes and substrates. Cellobiases (β-glucosidases) are measured by the hydrolysis of cellobiose or aryl β-glucosides such as salicin or *p*-nitrophenyl β-D-glucoside by generally accepted procedures since these substrates are soluble and well defined. Specific activities are about 150 IU/mg protein. Cellulose is a variable substrate depending on the source and pretreatment. Cellulase activities may be expressed, according to the substrate, as carboxymethyl cellulase, Walseth (phosphoric acid swollen) cellulase, filter paper cellulase, Avicelase, cotton cellulase, etc. For quantitative work, activities are expressed as international units (IU) which must be based on significant but equal conversion of the substrate since hydrolysis rates decline rapidly and initial rates are not meaningful.[51] Specific activities of cellulases are low, 10–25 units/mg protein for carboxymethylcellulase, ~0.6 units/mg protein for filter paper cellulase, and ~0.02 units/mg protein for cotton cellulase. The above assays are based on the production of reducing sugar or glucose. Endo-β-glucanases can also be measured by the reduction in viscosity of carboxymethylcellulose.[163] This assay is much more sensitive, since a random break in a cellulose chain may reduce viscosity by 50% but cause almost no increase in reducing groups. Some efforts have been made to improve the assay sensitivity[37,58] and to implement an automated system.[46]

Recently, new mutant strains producing as much as 80 IU/l/h in a batch culture system and 7 IU/g cell/h in a continuous culture system have been selected, and the strain improvement work continues in several laboratories (*Table 1*).

Considerable efforts have been focused on obtaining high cellulase producing organisms and/or mutants. Among these are the enhanced cellulase mutants from *T. reesei*,[32,57,76,78] thermophilic actinomyces,[69,73,79,85,92,94,118,154] thermophilic anaerobes,[45] yeasts,[75,111] and other mutant strains.[39,49,50,139]

Table 1 Cellulase production by mutant strains of *Trichoderma reesei*

Strain	CMC (units/ml)	Filter paper (units/ml)	β-Glucosidase (units ml)	Productivity (FPU/l/h)	Soluble protein (mg/ml)
QM6a (parent)	88	5	0.3	15	7
QM9414 (Natick)	109	10	0.6	30	14
MCG77 (Natick)	104	11	0.9	33	16
C30 (Rutgers)	150	14	0.3	42	19
NG14 (Rutgers)	133	15	0.6	45	21

Cultures grown 14 days in 10 l fermenters on 6% 2 roll-milled cotton pH control >3.0 using 2 N NH_4OH
Enzyme units = μmol glucose produced per min in standard assay

Due to substrate multiplicity in terms of the origin and widely varying degree of reactivity of the cellulosic substrate to hydrolysis, many physical and chemical pretreatment methods are being developed in order to improve the hydrolysis yield.[117,136,155] The properties of reactive substrates have been studied,[102–104,165] and the susceptibility of cellulosic substrates to cellulase examined.[101] The extent of the effect of the sources of cellulosic materials, whether agricultural or waste, on the hydrolysis has also been studied.[22,126,130,138,144,146,148,150,157–159,161] The continuous hydrolysis process of cellulose has been attempted by several laboratories.[114–116,131] Enhanced hydrolysis was achieved by the addition of supplemental β-glucosidase to the cellulose hydrolysis system.[164] The important variables involved in cellulose saccharification have been studied,[112,124,132,160] and the hydrolysis mechanism examined.[100,113,135,141–143,156,162]

The kinetics of hydrolysis have been studied using different approaches: from the cellulase adsorption viewpoint;[14,121,122,131,140] from the reaction mechanism viewpoint,[166] including inhibition,[119] deactivation,[120] and enzyme and substrate concentrations.[122] Some modelling has been attempted but this has not been entirely successful due to a lack of good understanding of the exact reaction mechanism of the cellulase complex.[121,128] Engineering aspects of cellulose hydrolysis have also been considered.[107] The question of lignin degradation and its susceptibility to enzymatic hydrolysis has seriously been tackled recently.[108,109,125,127,134,145]

The progress in cellulase productivity is rather impressive, especially within the last few years, and the increasing trend of enzyme productivity is anticipated to continue for some years to come.

An assessment of the economics of cellulase process technology is important in determining (a) the priority areas of research and development endeavours and (b) the economic feasibility of practical applications.

Recently, assessment studies were made by several groups, including private, academic, industrial, and government groups. However, the criteria and basis of their studies differed somewhat. This reflects a keen interest in alternate process technology for the production of liquid fuels and chemical feedstocks from renewable resources. Some of these economic evaluations include cellulase production,[12] ethanol production,[13,18,19] enzymatic hydrolysis,[14,15,17] and so on. The studies show that the production of enzyme is the most expensive part of the cellulase process technology. It is estimated that the production cost of cellulase is ~$3.00/kg cellulase protein having a specific activity of 0.6 IU/mg protein. This enzyme production cost varies with the carbon source used, and it could be lowered to ~$1.50/kg.

The production cost of glucose from cellulose by enzymatic hydrolysis is about \$0.15/kg crude glucose syrup, and that of ethanol is about \$1.43/gallon 95% ethanol based on the current cellulase process technology without taking any by-product credits (*Figure 1* and *Table 2*).[13]

In all aspects of process components, enzyme production, pretreatment of the cellulosic substrate, hydrolysis, ethanol fermentation, and ethanol concentration, significant process improvements and process cost reduction are anticipated in the near future. The improvement in cellulase productivity to a level of 150–200 IU/l·h, and an increase in the utilization efficiency of cellulase for cellulose hydrolysis to a level of about 60–80% from the current level of ~15% would make the cellulase technology very attractive and competitive with other alternative technologies applicable to production of renewable carbon and energy resources.

Sensitivity analysis shows that the carbon source used is the major cost factor in cellulase process technology. It is clear that a significant cost reduction must be achieved in order for the cellulase process technology to become commercially practicable.

The application of cellulase technology certainly has been drawing more attention than any other aspect. Many have tried to develop a broad-scope application for the production of fuels, chemicals, and food.[10,104,151,186,187,191] Some have concentrated their endeavours on the production of alcohol,[16,147,152,184,192,195,199,200,209] application to animal feeds, related products like single cell protein and effective methods of cellulose utilization[65,66,105,106,123,133,149,153,158,167,182,188,189,199,201,203–206] and on the production of chemicals. Some examples are acetone and acetate,[192] xylitol, xylan, and xylose,[137,198] glycine, glyoxalic acid, tartrate and other organic acids,[183,197] glycerol, ethylene glycol, sorbitol, propylene glycol, erythritol, xylitol, etc.,[207] furfural,[100,185] plastic polymers,[110] wood sugar chemicals,[194,202] chemicals from lignocellulose,[196] and chemicals from hemicellulose.[193]

Table 2 Relative cost factor analysis for production of ethanol from cellulose. Data from Spano *et al.*[13]

	Unit cost (¢/gal 95% ETOH)	Percent cost
Enzyme production	57.33	43.4
Pretreatment	30.38	23.0
Hydrolysis	13.03	10.0
Ethanol production	31.07	23.6
Total	131.81	100.0

There are indications that many carbon sources that can be derived from cellulose hydrolysis will be used as chemical feedstocks, including industrial fermentation, as soon as the process economics can be justified for such applications.[149] Many view this possibility as being only a question of time.

Cellulase production

Induction and regulation

Cellulase is an inducible enzyme system. Several carbon sources have been tested to find the best inducer.[50,53] Cellulose itself has been recognized as the best inducer for the complete cellulase complex. Other inducers include sophorose and lactose.[50,52,59] Cellulose, cellobiose, and lactose are effective only at high concentrations. Sophorose is active at very low concentrations.[96,97] The natural inducer which abolishes repression of cellulase biosynthesis has not been identified but studies show that cellulase biosynthesis is repressed by a glucose catabolite.[34,36,60,63,87] When glucose is pulse-fed to the culture where cellulase biosynthesis is in progress, the cellulase biosynthesis ceases immediately, presumably due to catabolite repression, until glucose is exhausted or its residual concentration falls below a critical level of ~0.1 mg/ml (*Figure 2*).[32,67] Although it has been reported by Berg[98] that *Trichoderma* cellulase is released by lysis, other recent studies suggest that induction, synthesis and secretion of cellulase are closely associated or are concurrent events occurring at the cell surface.[96] The cellulase produced by *T. reesei* with cellulose or lactose as the sole carbon source contains a complete and well-balanced cellulase complex, whereas some other carbon sources, like sophorose alone, give a less complete array of cellulase protein components.[96,97] However, all *T. reesei* cellulase preparations contain adequate levels of the necessary components to hydrolyse insoluble cellulose, regardless of the inducer. Nisizawa *et al.* found that the catabolite repression of biosynthesis of inducible cellulase occurs at the translation level based on their studies with actinomycin D and puromycin.[61] They also found that the assimilation of amino acids like L-leucine is closely related to the presence or absence of an inducer like sophorose, and this finding suggests that the regulation of cellulase biosynthesis is also closely related to amino acid assimilation and metabolic regulation.[59] Mutant strains developed to date have similar patterns of cellulase enzymes when grown in the same medium, and also respond similarly to inducers and fermentation conditions. Under optimal conditions enzyme yields

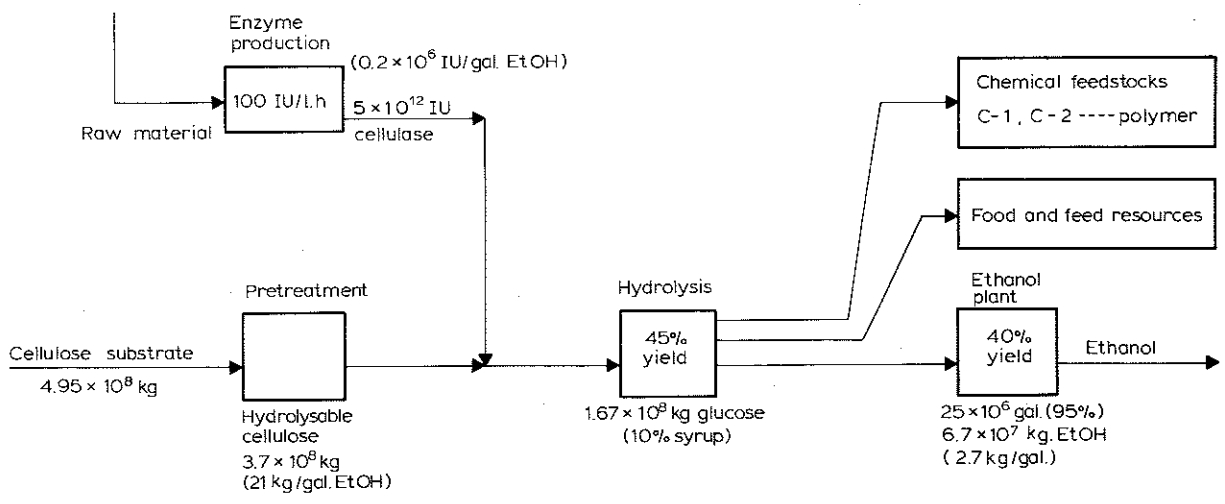


Figure 1 Process flowsheet and overall material balance for production of ethanol (25 × 10⁶ gal/yr) from cellulose (Spano *et al.*[13])

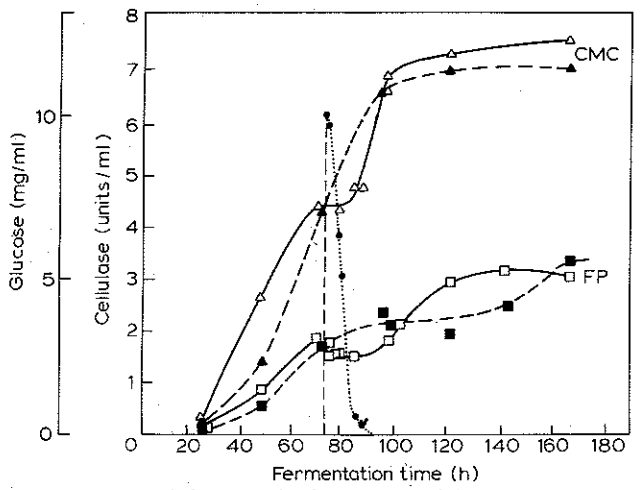


Figure 2 Effect of glucose pulse feeding on cellulose biosynthesis (Gallo *et al.*[32]) △, ▲, CMC; □, ■, FP; ●, G

of 5–15 International Filter Paper Units per ml (IFPU/ml) are obtained, which corresponds to 7–21 mg soluble protein per ml. The difference between the wild strain and the best current mutant is about three-fold (*Table 1*).

Cellulase fermentation

The medium for *T. reesei* culture has been developed and optimized for the maximal enzyme production using batch cultures.[50,80] The yield constants for cell growth with respect to carbon, oxygen, and nitrogen have been determined and reported, but those with respect to other growth factors or minerals have not been determined. *T. reesei* strain grows well in a synthetic medium containing lactose, ammonium sulphate and minerals.

Enzyme production, measured as the final cellulase concentration in the fermentation broth, is almost proportional to the initial cellulose concentration, up to 8% cellulose, in a batch culture where cellulose is used as the sole carbon source (*Figure 3*).[80] Cellulase biosynthesis seems to require residual inducer in the culture medium for a period of ~30–50 h when cellulose, lactose, or glucose and sophorose is the growth substrate[28,32,68,70] used, whereas the induction time is reduced to ~2 h when sophorose is used with washed mycelium.[96]

The maximum growth rate of *T. reesei* mutants in synthetic medium varies within the range of 0.1–0.25 h^{-1}, depending on the carbon source and nitrogen source used. *T. reesei* strain grows rapidly on simple sugars like glucose and fructose and rich nitrogen source like peptone, but more slowly with a lag on lactose or cellulose and ammonium salts. It grows well on ammonium sulphate, ammonium phosphate, and ammonia, but is unable to utilize nitrate. It can assimilate a wide range of carbon sources. Complex nitrogen sources containing amino acids stimulate growth and enzyme production.

The optimal pH for growth is ~4.0 and that for enzyme production is ~3.0. Below pH 3.0 the cellulases are inactivated, and growth ceases below pH 2.0. Control of pH within the range of 3.0–4.0 is very important to maximize enzyme production during the enzyme production phase.

The optimal temperature for growth is ~32–35°C, and that for enzyme production is ~25–28°C. Programmed temperature and pH profiles could be used to optimize both cell growth and cellulase production in a batch culture system.

Most cellulase is released into the fermentation broth after the residual reducing sugar concentration falls below the critical level of 0.1 mg/ml and after growth reaches the maximum cell concentration level (*Figure 4*). The amount of cellulase which accumulates inside the cell during fermentation is insignificant compared to the amount of extracellular cellulase released into the broth as soluble protein. In some cases the formation of as much as 20 mg cellulase protein/ml in the form of soluble protein has been observed.

An adequate oxygen transfer rate in the fermentation broth must be maintained during growth and enzyme production. During the enzyme production phase, however, there is a requirement for at least the maintenance level of oxygen uptake rate for energy metabolism, and the corresponding amount of oxygen must be supplied to the cells. If the oxygen transfer rate is insufficient or if it is less than the maintenance ration of oxygen, cellulase biosynthesis ceases.

The interactions among the fermentation variables are recognized as very complex, and further process improvement will require systematic optimization studies on the effects and interactions of important fermentation process variables. An example of such a study is illustrated in the next section, which describes the quantitative physiology of *T. reesei*.

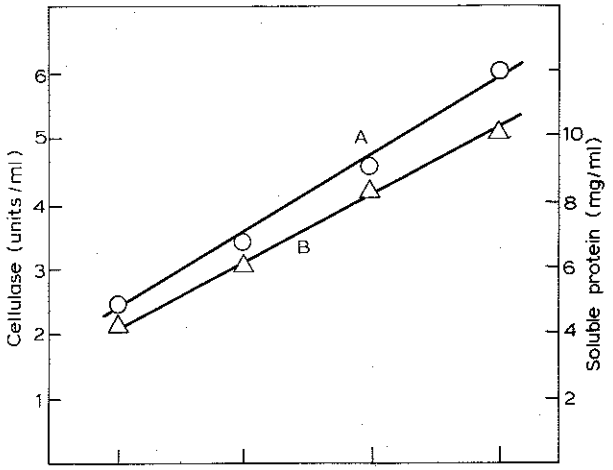


Figure 3 Effect of cellulose concentration in the medium on production of cellulase (A) and soluble protein (B) by *Trichoderma reesei* QM9414 (Sternberg and Dorval[80])

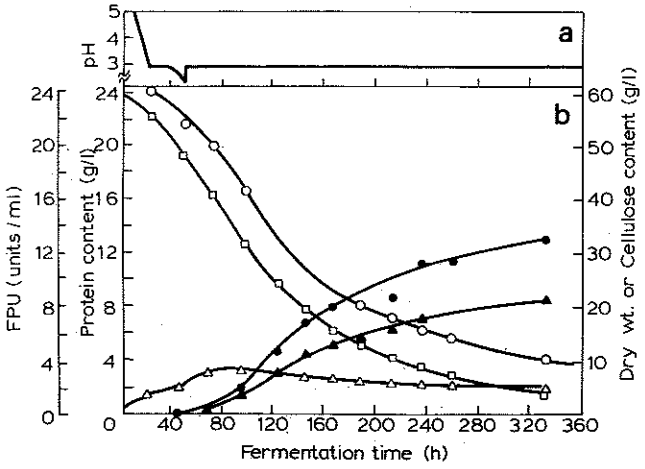


Figure 4 Typical fermentation profile with QM9414 strain showing concentration of (a) pH, and (b) enzyme, cell concentration and soluble protein. ○, Dry wt.; □, cellulose; ●, soluble protein; △, mycelial protein (g/l); ▲, FPU (IU/ml)

Quantitative physiology

Recent studies employing a two-stage continuous culture system have successfully separated the growth and enzyme production stages and evaluated the physiological parameters involved in growth and cellulase biosynthesis.[70] To determine the specific rates accurately, a synthetic medium containing only lactose, ammonium sulphate and mineral salts was used. Two different sets of optimal conditions for cell growth and enzyme production were imposed on each stage.

Using such a system, the specific rates of uptake of carbon, oxygen and nitrogen were determined. The values of these specific uptake rates and yield constants are presented in *Table 3*.[70] The maintenance coefficients and yield constants for carbon and oxygen are the same for both stages. The yield constants for nitrogen in the growth and enzyme production stages were found to be different. This reflects the fact that the protein content (50%) in the growing cells in the first stage is significantly higher than that (38%) in the cells producing enzymes in the second stage. This difference in protein content in the cells could be accounted for partly by the enzyme protein released by the cells into the broth in the second stage. The specific uptake rate of nitrogen at zero specific growth rate in the second stage represents an additional amount of nitrogen incorporated into the enzyme protein synthesized and released in the second stage.

An important finding in this study was that maximal specific enzyme productivity could be achieved when the specific growth rate was maintained at about zero in the second stage. While the specific growth rate was kept at or near zero in the enzyme production stage by means of an adequate feed rate of carbon or oxygen, the dilution rate could be varied and the optimal dilution rate could be determined. The optimal dilution rate in the second stage was found to be ~0.027 h^{-1}, which corresponds to ~37 h mean residence time. This period of mean residence time of cells in the second stage must be required, apparently, for induction, biosynthesis and release of cellulase into the fermentation broth.

Based on these results from the studes on quantitative physiology, the cellulase fermentation process may be designed and controlled for the purposes of process improvement and increasing enzyme productivity.

Using the specific uptake rates and yield constants determined for a given strain, the optimal rates of carbon and nitrogen feedings and the oxygen transfer can be estimated and used as the process control parameters for maximal enzyme productivity. As an example, the optimal rates of carbon, nitrogen and oxygen supply are calculated and are shown in *Table 4*. These optimal specific feed rates take into account nutrient requirements for biomass, maintenance or energy metabolism, and enzyme production. The conditions and data used for this calculation are: cell concentration in both the first and second stages, 10 g/l; specific growth rate in the first stage, $\mu_1 = 0.1\ h^{-1}$, and that in the second stage, $\mu_2 = 0$; specific enzyme productivity in the second stage, $Q_2(E) = 8$ IU/g cell · h; dilution rate in the second stage, $D_2 = 0.03\ h^{-1}$; and the metabolic rate data as shown in *Table 3*.

The optimal feed rates will vary for other mutant strains with different physiological activity, metabolic rates and enzyme productivity. Thus, new sets of optimal feed rates will have to be estimated for process design and for the control of new mutant strains.

The yield factors for enzyme production with respect to carbon, nitrogen, and oxygen can also be estimated on the basis of these specific feed rates and the specific activity of the enzyme. An example is illustrated in *Table 5*. Once these yield factors for enzyme production are determined and the unit cost of nutrient sources is known, the raw material cost for the process technology can be estimated readily.

Table 3 Metabolic constants for *Trichoderma reesei* MCG77 on lactose (based on two-stage continuous culture data. Ryu *et al.*[70])

	Oxygen	Carbon	Nitrogen	
Parameters	Stage 1 = Stage 2	Stage 1 = Stage 2	Stage 1	Stage 2
M (mg/g biomass/h)	27.0	10.0	0.0	0.9
M (mmol/g biomass/h)	0.85	0.83	0.0	0.064
Y (g biomass/g)	1.0	1.1	12.5	16.6
Y (g biomass/mol)	32.0	13.0	175.0	232.0

M = maintenance coefficient; *Y* = yield constant; Stage 1 = growth; Stage 2 = enzyme production

Table 4 Estimation of specific feed rates of nutrients for cellulase production

Nutrient sources	Stage 1	Stage 2	Total
Carbon (mg C/g cell · h)	100	16	116
Oxygen (mmol O_2/g cell · h)	4	1	5
Nitrogen (mg N/g cell · h)	8	1	9

Table 5 Yield factors for enzyme production

Nutrient sources	Amount of enzyme produced per unit amount of nutrient consumed	Amount of nutrient required per unit amount of enzyme produced
Carbon	112.5 IU/g C	8.9 mg C/IU
Oxygen	2600 IU/m O_2	0.38 mmol O_2/IU
Nitrogen	1610 IU/g N	0.62 mg N/IU

Enzymatic hydrolysis

Reese *et al.*[143] began their studies on the mechanism of enzymatic hydrolysis of cellulose, and they proposed a multienzyme system, C_1 and C_x cellulase complex. Since then, many groups have separated the cellulase components, and attempts have been made to purify and characterize the cellulase components.

Although the detailed reaction mechanisms and synergistic interactions involved in enzymatic hydrolysis of cellulose have not been completely elucidated, the pattern is now understood to be based on action of endo-β-glucanases, exo-β-glucanases and cellobiases. A simplified reaction and the mode of action of cellulase complex are shown in *Figure 5*.

Many have concentrated their efforts on separation, purification, and characterization of cellulase components.[24,33,76,91,93] As many as 12 cellulase components have been identified and their synergistic effects have been reported.[162]

The cellulase complex of *T. reesei* contains several enzyme components[91] that act synergistically in the hydrolysis of insoluble cellulose (*Table 6*). Reese postulated the

Table 6 Relative cellulase activities of the components of *Trichoderma koningii* cellulase alone and in combination, as a synergistic effect (Wood and McCrae[163])

Enzyme	Relative cellulase activity (%)
C_1	<1%
$C_X - 1$	<1
$C_X - 2$	<1
β-Glucosidase – 1	nil
β-Glucosidase – 2	nil
C_1 + β-Glucosidase (1 + 2)	5
C_X (1 + 2) + β-Glucosidase (1 + 2)	4
C_1 + C_X (1 + 2) + β-Glucosidase (1 + 2)	103
Original culture filtrate	100

Cellulase activity = Hydrolysis of cotton. Components added at level equal to original filtrate

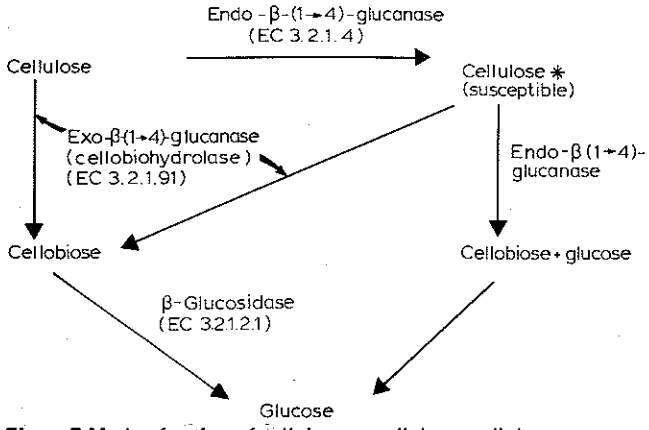
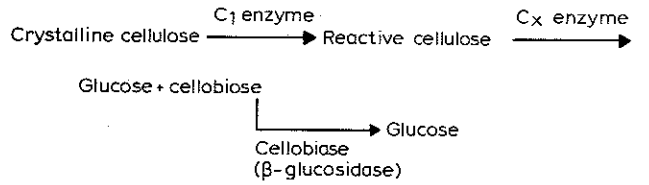


Figure 5 Mode of action of cellulase on cellulose–cellulase enzyme system

multiplicity of cellulase and introduced the C_1 and C_X hypothesis to explain the differences between what we now call complete and incomplete cellulases.[143]



This concept has been a great stimulus to cellulase research even though it is an oversimplification. A great deal more work has been done, particularly by Halliwell,[176] King,[178] Nisizawa,[179] Wood,[91,93,162] Pettersson,[141,142,168,174,175,177] Brown,[21,27,169–173] and Tsao[33] on the separation, purification and characterization of the cellulase components of *Trichoderma*. The principal enzymes include endo- and exo-β-glucanases (cellulases) and β-glucosidases (cellobiases) (*Figure 5*).

Endo-β-glucanases are cellulases that hydrolyse the chains in a random fashion, resulting in a rapid reduction in chain length or the degree of polyermization together with a slow increase in reducing groups. The β-glucose configuration is retained and the final product is a mixture of glucose and cellobiose. Exo-β-glucanases are cellulases that remove glucose or cellobiose units from the non-reducing end of the chain, resulting in a rapid increase in reducing groups with little change in fluidity. Sugars are released in the α-configuration (inversion). The best known exo-β-glucanase is the cellobiohydrolase of *Trichoderma*, which gives cellobiose as the sole product. Cellulases hydrolyse cellodextrins more rapidly as chain length increases. They are specific for β(1 → 4)-glucosidic linkages between unaltered glucose units, and are strongly inhibited by methyl cellulose. The cellobiase of *Trichoderma* is a β-glucosidase, it acts also on aryl-β-glucosides and is inhibited by nojirimycin and gluconolactone. The rate of hydrolysis of cellodextrins increases as chain length decreases. Cellobiase is specific for β-glucosides but does not require glucose as the aglucone, and will hydrolyse β(1 → 2)-, β(1 → 3)-, and β(1 → 6)-linkages as well as the β(1 → 4)-linkage. The configuration is retained on hydrolysis. These enzymes can also be transferases acting on glucose units and forming alcohols, or other sugar molecules such as dimers, trimers, and higher oligosaccharides.[141]

Cellulose has a fairly simple chemical structure. It is an unbranched polymer containing glucose units linked only by the β(1 → 4)-glucosidic bonds which are hydrolysed by the cellulase components. Because of the β-configuration all of the hydroxyl bonds are in the same plane. Therefore, in a cellulose microfibril where the chains are ordered, powerful hydrogen bonding occurs, resulting in a crystalline structure. This gives cellulose its insolubility, tensile strength, and resistance to enzymes and chemical reagents. Where chains are less ordered or frayed the cellulose is amorphous, more readily hydrated and more accessible to enzymes. Such amorphous areas can be hydrolysed to some extent by individual endo- or exo-glucanases acting alone, or by incomplete cellulases. For hydrolysis of crystalline cellulose where hydrogen bonds as well as glucosidic bonds must be broken a complete cellulase containing an adequate level of all the necessary components is required.[132]

Extensive hydrolysis requires a susceptible substrate as well as a complete cellulase. When the sugar produced during enzymatic hydrolysis of different cellulose substrates is plotted against time, curves showing a similar pattern of a rapid initial rate followed by a declining rate are obtained, although the rate and extent of saccharification are greater for a susceptible substrate such as ball-milled pulp than for more resistant substrates such as hammermilled pulp or microcrystalline cellulose. Attempts to explain these kinetics have been based on substrate multiplicity, product inhibition, enzyme inactivation, enzyme adsorption and variation in the association constant as hydrolysis proceeds (*Table 7*). We believe that all these factors are important and that they

Table 7 Problems related to enzymic saccharification of cellulose, and possible solutions

Problems	Solutions
Require complete cellulase	Strain selection
Endo β-glucanases	*Trichoderma reesei* for cellulase
Exo β-glucanases	*Aspergillus phoenicis* for supplemental β-glucosidase
Other (xylanase, etc.)	
Low specific activity of cellulase	Increase enzyme productivity
Must produce high soluble protein	Optimize fermentation
Glucose repression	Mutation for derepression
Cellulose induction	Constitutive mutant?
Decline of hydrolysis with time	Study enzyme–substrate interactions
Increasing substrate resistance	Pretreatment
Product inhibition	Add supplemental β-glucosidase, simultaneous saccharification and ethanol fermentation
Enzyme inactivation	Mutation/chemical stabilization

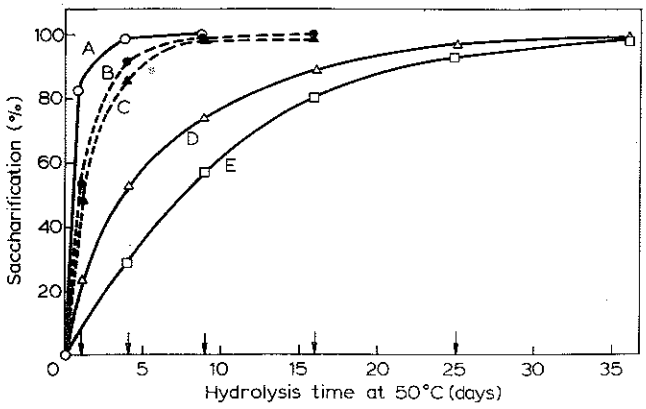


Figure 6 Enzymatic hydrolysis of pure cellulosic substrates of varying resistance showing substrate multiplicity. Conditions: 10% substrate, 2 FPU/ml; pH 4.8; 50°C. A, Farrel Birmingham (FB) two-roll mill Solka Floc; B, ball milled pulp (BW200); C, Solka Floc pulp (SW40); D, FB cotton; E, cotton. ○, Roll milled SW40; ●, BW200; ▲, SW40 (pulp); △, roll milled cotton; □, cotton; ↓, centrifuge down solids, resuspend in fresh enzyme

interact to enhance each other, particularly for enzymes acting on crystalline (resistant) substrates. The most important factor affecting the rate and extent of hydrolysis is substrate multiplicity (*Figure 6*). Amorphous cellulose is hydrolysed much more rapidly than crystalline cellulose. The quantity of amorphous cellulose can be increased by pretreatments such as pulping or milling resulting in an increase in the rate and extent of hydrolysis. Native cellulose is usually in close association with non-cellulosic materials such as hemicelluloses and lignins. Hemicelluloses are often easily degraded by xylanases, mannanases etc. which are present in some cellulase preparations, so that their presence may actually lead to increased production of reducing sugars and greater susceptibility of the residual cellulose. Lignins are not degraded and create many problems, preventing access of enzyme to substrate, adsorbing enzyme in inactive complexes, and occupying valuable reactor space.

The second factor is product inhibition. Saccharification syrups contain not only glucose and cellobiose but also other sugars, such as xylose.[64] Cellulase is moderately inhibited by glucose and strongly inhibited by cellobiose.[132] For both sugars the extent of inhibition increases with increasing resistance of the cellulose. Cellobiase is inhibited by glucose.[26,33] The inhibitions are competitive. As hydrolysis proceeds, cellobiose accumulates. *Trichoderma reesei* cellulase preparations have ~0.2–0.3 cellobiase units per filter paper cellulase unit. This is sufficient to hydrolyse the cellobiose produced during growth on cellulose where sugars do not accumulate. In a saccharification reactor, however, glucose does accumulate, the ratio of glucose to cellobiose is high and cellobiase is inhibited. The resulting build-up of cellobiose inhibits the cellulase. Therefore, the addition of supplemental cellobiase, an enzyme which in itself has no action on cellulose, is remarkably stimulatory to cellulase.[26] To date, the efforts to increase the cellobiase level in *T. reesei* cellulase preparations by mutation or process improvement have not been very successful. A good source of cellobiase is *Aspergillus phoenicis*. Addition of the *Aspergillus* cellobiase to *Trichoderma* cellulase reduces cellobiose and greatly increases glucose and total sugar in the digest. A maximum effect is seen at a ratio of ~1.5 cellobiase units per filter paper cellulase unit.[8] Since cellobiose is a soluble substrate, it is desirable to immobilize the enzyme so it can be reused. The enzyme has been successfully immobilized on chitosan using the bifunctional agent glutaraldehyde. Compared to the free cellobiase, the immobilized enzyme has a similar pH optimum, ~4.5, but increased activity at lower pH values, and improved thermal stability.[26] The immobilized enzyme has been sandwiched between stainless steel mesh for use in the saccharification reactor. It works as well as the free enzyme and can be reused several times without significant loss of activity.[26]

Fortunately, *T. reesei* cellulase is fairly stable to heat (50°C), and many chemicals. The enzymes are glycoproteins with no cofactor or metal requirements. Broths can be kept frozen or refrigerated for years, or the enzyme can be freeze-dried or precipitated by acetone or ethanol, all without serious loss of activity. Saccharifications are carried out in stirred tank reactors for 24—48 h or longer. However, the rate of hydrolysis decreases rapidly with time and the more resistant the substrate the greater the decrease in rate. This is due to inactivation of the enzymes or complexes of enzymes required for the hydrolysis of crystalline cellulose.[132,181] When cellulase was held for 28 days at pH 4.8 and 50°C (the optimum conditions for saccharification), in the absence of substrate, protein, β-glucosidase (Salicinase) and endo-β-glucanase (CMCase) were stable, but activity on filter paper fell to 50% and on cotton to less than 20% in two days. The residual activity reflects the stability of components that can hydrolyse the more susceptible portions of these substrates. Nevertheless, the residual enzyme after 28 days at 50°C converted 79% of a 5% slurry of a susceptible substrate (milled cellulose) in 24 h, yielding 4% syrup. Control enzyme held in the refrigerator was only 5% better, giving 84% conversion under the same conditions.[132,181]

So the rate and extent of saccharification depend on the nature and pretreatment of the substrate, enzyme and substrate concentration (i.e. the enzyme/substrate ratio) product inhibition, and enzyme stability. All of these interact to cause the rate of hydrolysis to fall off rapidly with time. As the more susceptible portions of the cellulose are hydrolysed, the residue is increasingly crystalline and resistant, products accumulate and they competitively inhibit the enzymes. Since cellulose simultaneously decreases, the inhibitor/substrate ratio rises. Furthermore, cellobiose inhibition is

Table 8 Selected methods of pretreatment of cellulosic materials to enhance enzyme susceptibility

Type	Advantage	Disadvantage
Physical		
Hammermill	Less costly	Ineffective
Colloid mill	Improves handling	
Ball mill	Effective	Energy-intensive
Two-roll mill	High bulk density	Expensive
Steam explosion	Effective	Low bulk density
220–240°C, 600 lb/in.²	Less costly	Ineffective for softwoods
Alkali swelling NaOH	Moderately effective	Low bulk density
	Less costly	Must wash or neutralize
Delignification		
Chemical pulping	Moderately effective	Expensive
Biological	Conserve enzyme and reactor space	Low bulk density Pollution
Dissolve, reprecipitate		
Cadoxen (toxic)		Low bulk density
Hydrazine (toxic)		Expensive
Ferric tartrate NaOH	Effective	Difficult handling
Cuprammonium		Solvent recovery
Viscose		Side reactions
Concentrated acid		Pollution

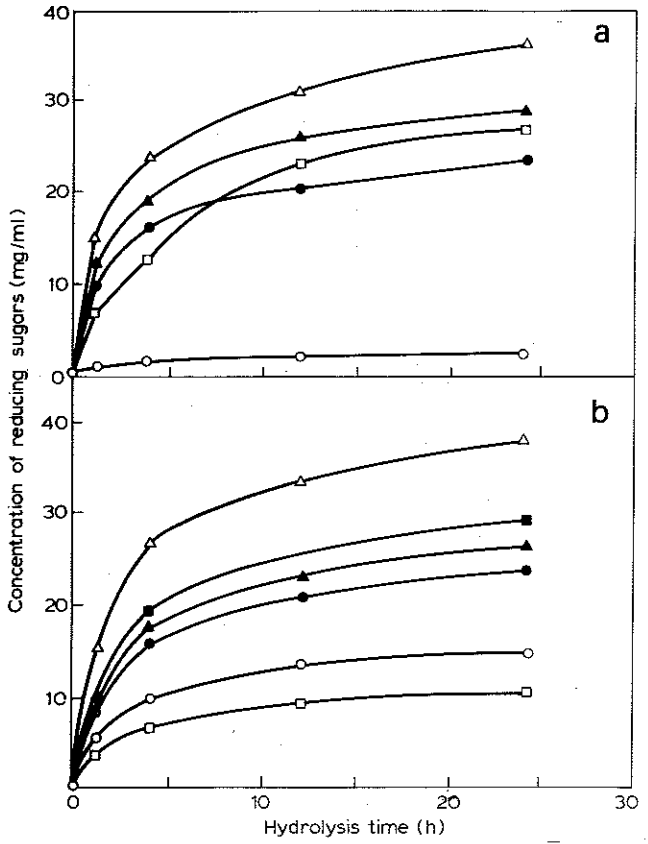


Figure 7 Pretreatment of hardwood (poplar) compared with softwood (newspaper). Hydrolysis at 50°C, pH 4.8, with *T. reesei* cellulase at 1 FP unit/ml. TRM = two-roll (compression) milled; solvent = cuprammonium reprecipitated (Tassinari and Macy[95]). (a) 5% Popular shavings: △, TRM (6 min); ▲, TRM (3 min); □, steam (190°C); ●, attritor (30 min); ○, control. (b) 5% Newspaper: △, TRM (10 min); ■, solvent; ▲, TRM (4 min); ●, attritor (30 min); ○, control; □, steam (200°)

much more severe when substrates are crystalline. Finally, enzymes are inactivated under the reaction conditions and those enzymes or complexes of enzymes acting on crystalline substrates are inactivated the most rapidly. Nevertheless, our present broths can carry out extensive saccharifications of many cellulose substrates.[130]

Pretreatment is required for most substrates. For example, ball milling reduces particle size thereby increasing bulk density, available surface, and reactivity. Most importantly it also reduces crystallinity. When milled and unmilled cellulose of the same particle size (400 mesh) are compared, the milled cellulose is more reactive.[130] A number of effective chemical and physical pretreatments are known (*Table 8* and *Figure 7*). New pretreatments that have attracted much recent attention include compression (two-roll) milling[155] and dissolving and reprecipitating the cellulose.[166] Both are certainly effective but the economics are not yet clear. Steam explosion is a more economical pretreatment and is effective on hardwoods and agricultural wastes, but not on softwoods. Further research is needed to select the optimum conditions and best pretreatments for different substrates, based on effectiveness, economy and energy utilization.

Discussion

There are many research and development problems related to cellulase process technology, and a great deal more work needs to be done before its practical applications are realized. The methods of production, harvesting, collection, conservation and management of biomass should be improved to ensure an adequate supply of renewable carbon source raw materials. Cellulase productivity must be improved further through strain mutation and optimization of the fermentation process. Induction and regulation of cellulase biosynthesis must be better understood if it is to be fully exploited for increased cellulase productivity.

The fundamental problems involved in the enzymatic hydrolysis of cellulose are in three main areas.

(i) Solid substrate and substrate multiplicity—lignin and hemicellulose content, size and surface area of cellulosic substrate material, crystallinity and degree of polymerization, degree of adsorption of the enzyme on cellulose and lignin, and the association constant between cellulose substrate and enzyme all vary with the substrate source and the time during the hydrolysis reaction.

(ii) Multienzyme system – there are several cellulase components, including endo- and exo-cellulases and β-glucosidase, and they have a well-coordinated and concerted reaction mechanism by which the polymeric cellulosic substrate is degraded to glucose monomer. In the hydrolysis reaction sequence, the product of each reaction step inhibits the enzyme. The effect of product inhibition on the β-glucosidase is most severe, due to accumulation of final product in the reaction sequence. The relative activity and rate of each enzyme component should be optimized with respect to the overall reaction rate in order not to cause any rate-limiting step in the sequence of reactions.

(iii) Enzyme inactivation – loss of enzyme activity and enzyme deactivation are caused by factors such as heat, pH, shear, mineral ions, proteases, and cellulase adsorbents, including cellulose and lignocellulose. The degree of cellulase deactivation by these factors varies with different cellulase components during hydrolysis.

More refined work on the purification, separation, and characterization of cellulase components should be carried out in order to further our understanding of the basic hydrolysis reaction mechanisms, kinetics, and interactions of cellulase components. More meaningful modelling and simulation work could be carried out using a good understanding of the reaction mechanisms and kinetics of cellulose hydrolysis.

The design, control and optimization of the cellulase reactor system should be studied to increase productivity and improve the reactor performance. The arrangement and configuration of cellulase reactor systems consisting of one or more reactors containing combined or individual enzyme components are such examples.

Although the subject is outside the scope of this paper, novel methods for producing low molecular weight chemical feedstocks should actively be explored using chemical, enzymatic or biological methods, or combinations of these methods. It is highly likely that large portions of the chemical feedstocks now available only from petroleum downstream products may be replaced in the foreseeable future by those from cellulosic materials, and these may reduce petroleum requirements significantly.

Another very important area related to cellulase technology is the development of economical process technology for the production of liquid fuels primarily for transportation purposes. Although alcohol production from simple sugars is a well established technology, further process improvement in alcohol fermentation and recovery will reduce the production cost of alcohol significantly. For example, yeast fermentation to ethanol has been combined

with the hydrolysis step with moderate success.[147,195,200,208] *Saccharomyces* and *Candida* yeasts are compatible with the cellulase enzymes and the saccharification syrups. Ethanol is less inhibitory to the cellulase enzymes than equivalent quantities of glucose or cellobiose so that under equal conditions saccharification is greater in the coupled system. This also solves the difficult problem of preventing contamination in the saccharification reactor without the addition of toxic or inhibitory chemicals. The coupling of the two systems also reduces capital costs. However, disadvantages exist. The coupled system must be optimized for the yeast fermentation, which requires that temperatures be reduced to well below the optimum for enzyme hydrolysis. Although *Candida* yeasts can grow aerobically on xylose, *Saccharomyces* will not. Neither yeast can ferment xylose to ethanol. Well over 200 basic organic chemicals can be obtained from ethanol and ethylene, which is in turn derived from cellulosic materials.[20]

References

1 *Symposium on Enzymatic Hydrolysis of Cellulose* (Bailey, M., Enari, T. M. and Linko, M., eds) The Finish National Fund for Research and Development (SITRA), Helsinki, 1975
2 *Enzymatic Conversion of Cellulosic Materials: Technology and Applications, Biotechnol. Bioeng. Symp. No. 6* (Gaden, Jr, E. L., Mandels, M. H., Reese, E. T. and Spano, L. A., eds) John Wiley and Sons, New York, 1976
3 *Bioconversion of Cellulosic Substances into Energy, Chemicals and Microbial Proteins* (Ghose, T. K., ed.) Indian Institute of Technology, Delhi, India, 1977
4 *Cellulases and Their Applications, Advances in Chem. Series 95* (Hajny, G. J. and Reese, E. T., eds) American Chemical Society, Washington, D.C., 1969
5 *Advances in Enzymatic Hydrolysis of Cellulose and Related Materials* (Reese, E. T., ed.) Pergamon, New York, 1963
6 *Biotechnology in Energy Production and Conservation, Biotechnol. Bioeng. Symp. No. 8* (Scott, C. D., ed.) John Wiley and Sons, New York, 1978
7 *Proceedings of 2nd Annual Symposium on Fuels from Biomass* (Shuster, W. W., ed.) Rensselaer Polytechnic Institute, Troy, N.Y., 1978
8 Han, Y. W. and Smith, S. K. *Utilization of Agricultural Crop Residues,* US Dept. Agriculture, Agricultural Research Service, ARS W-53, 1978
9 *Cellulose as a Chemical and Energy Resource, Biotechnol. Bioeng. Symp. No. 5* (Wilke, C. R., ed.) John Wiley and Sons, New York, 1975
10 Allen, W. G. in *Enzymatic Conversion of Cellulosic Materials: Technology and Application Biotechnol. Bioeng. Symp. No. 6* (Gaden, Jr, E. L., Mandels, M. H., Reese, E. T. and Spano, L. A., eds) John Wiley and Sons, New York, 1976, p. 303
11 Mandels, M. and Andreotti, R. E. *Process Biochem.* 1978, **13**, 6
12 Nystrom, J. M. and Allen, A. L. in *Enzymatic Conversion of Cellulosic Materials: Technology and Applications, Biotechnol. Bioeng. Symp. No. 6* (Gaden, Jr, E. L., Mandels, M. H., Reese, E. T. and Spano, L. A., eds) John Wiley and Sons, New York, 1976, p. 55
13 Spano, L., Allen, A., Tassinari, T., Mandels, M. and Ryu, D. in *Proceedings of 2nd Annual Symp. on Fuels from Biomass* (Shuster, W. W., ed.) John Wiley and Sons, New York, 1978
14 Wilke, C. R. and Mitra, G. in *Cellulose as a Chemical and Energy Resource, Biotechnol. Bioeng. Symp. No. 5* (Wilke, C. R., ed.), John Wiley and Sons, New York, 1975, p. 253
15 Wilke, C. R., Yang, R. D. and Von Stockar, U. in *Enzymatic Conversion of Cellulosic Materials: Technology and Applications, Biotechnol. Bioeng. Symp. No. 6* (Gaden, Jr, E. L., Mandels, M. H., Reese, E. T. and Spano, L. A., eds) John Wiley and Sons, New York, 1976, p. 155
16 Wilke, C. R., Cysewski, G. R., Yang, R. D. and Von Stockar, U. *Biotechnol. Bioeng.* 1976, **18**, 1315
17 Wilke, C. R., Yang, R. D. and Von Stockar, U. in *Preliminary Cost Analyses for Enzymatic Hydrolysis of Newsprint,* Lawrence Berkeley Laboratory, University California, Berkeley, 1975, Report No. 18 Conf. 750992–2, p. 44
18 *Comparative Economic Assessment of Ethanol from Biomass* US Department of Energy and the Mitre Corporation, 1978
19 *The Report on the Alcohol Fuels Policy Review* US Department of Energy, 1979
20 *Chemical Origins and Markets* Stanford Research Institute, 1974
21 Brown, D. E. and Halsted, D. J. *Biotechnol. Bioeng.* 1975, **17**, 1199
22 Amsallem, B. *Int. Biodeterior. Bull.* 1970, **6**, 135
23 Andreotti, R. E., Mandels, M. and Roche, C. in *Bioconversion of Cellulosic Substances into Energy, Chemicals and Microbial Proteins* (Ghose, T. K., ed.) Indian Institute of Technology Delhi, India, 1977, p. 249
24 Berghem, L. E. R., Petterson, L. G. and Axio–Fredriksson, U. B. *Eur. J. Biochem.* 1976, **61**, 621
25 Bernat, J. A. and Rzedowski, W. *Przem. Ferment. Rolny* 1977, **21**, 11
26 Bissett, F. and Sternberg, D. *Appl. Env. Microbiol.* 1978, **35**, 750
27 Brown, D. E. *Octagon Paper* 1976, **3**, 1
28 Brown, D. E. and Zainudeen, M. A. *Biotechnol. Bioeng.* 1977, **19**, 941
29 Bruchmann, E. E., Kirsch, B. and Lauster, M. *Chem. Ztg.* 1975, **99**, 157
30 Demain, A. L. *J. Appl. Chem. Biotechnol.* 1972, **22**, 345
31 Ferrer, A., Alroy, Y. and Brito, I. *Proc. Annu. Biochem. Eng. Symp.* 1977, **7**, 83
32 Gallo, B. J., Andreotti, R., Roche, C., Ryu, D. and Mandels, M. in *Utilization of Agricultural Crop Residues* (Han, Y. W. and Smith, S. K.) US Dept. Agriculture, Agricultural Research Service, ARS W-53, 1979, p. 89
33 Gong, D. S., Ladisch, M. R. and Tsao, G. T. *Biotechnol. Bioeng.* 1977, **19**, 959
34 Goldberger, R. F., Deeley, R. G. and Mullinix, K. P. *Adv. Genet.* 1976, **18**, 24
35 Highley, T. L. *Mater. Org.* 1976, **11**, 33
36 Holzer, H. *Trends Biochem. Sci.* 1976, **1**, 178
37 Huang, J. S. and Rang, J. *Anal. Biochem.* 1976, **73**, 369
38 Janicki, J., Gembicka, D. and Chrapkowska, K. J. *Pr. Kom. Nauk. Roln. Kom. Nauk. Lesn., Poznan. Tow. Przyj. Nauk.* 1976, **41**, 117
39 King, N. J. and Smith, G. A. *Int. Biodeterior. Bull.* 1973, **9**, 87
40 Komura, I., Awao, T. and Yamada, K. Jpn Kokai 77 134 090 (1977)
41 Komura, I. and Yamada, K. Jpn, Kokai 78 38 689 (1978)
42 Korculanin, A., Juric, Z., Pospisil, O. and Matanic, H. *Mikrobiologija* 1975, **12**, 81
43 Korculanin, A., Biocic, I. and Juric, Z. *Acta Bot. Croat.* 1977, **36**, 87
44 Lai, M. *Shih Pin Kung Yeh* 1977, **9**, 20
45 Lee, B. H. and Blackburn, T. H. *Appl. Microbiol.* 1975, **30**, 346
46 Leisola, M. and Kauppinen, V. *Biotechnol. Bioeng.* 1978, **20**, 837
47 Linko, M., Markkanen, P., Bailey, M. and Leisola, M. in *Symposium on Enzymatic Hydrolysis of Cellulose* (Bailey, M., Enari, T. M. and Linko, M., eds) The Finnish National Fund for Research and Development (SITRA), Helsinki, 1975, p. 329
48 Lobanok, A. G., Edzins, I., Bogomazova, L., Smetanin, V. V., Karklins, R. and Bekers, M. *Latv. PSR Zinat. Akad. Vestis* 1977, 27
49 Loginova, L. G., Guzhova, E. P., Ismailova, D. Yu and Burdenko, L. G. 1978, **14**, 485
50 Mandels, M. in *Cellulose as a Chemical and Energy Resource, Biotechnol. Bioeng. Symp. No. 5* (Wilke, C. R., ed.) John Wiley and Sons, New York, 1975, p. 81
51 Mandels, M., Andreotti, R. and Roche, C. in *Enzymatic Conversion of Cellulosic Materials: Technology and Application, Biotechnol. Bioeng. Symp. No. 6* (Gaden, Jr, E. L., Mandels, M. H., Reese, E. T. and Spano, L. A., eds), John Wiley and Sons, New York, 1976, p. 21
52 Mandels, M., Parrish, F. W. and Reese, E. T. *J. Bacteriol.* 1962, **83**, 400
53 Mandels, M. and Reese, E. T. *J. Bacteriol.* 1960, **79**, 816
54 Mandels, M. and Weber, J. *Adv. Chem. Ser.* 1969, **95**, 391

55 Mitra, G. and Wilke, C. R. *Biotechnol. Bioeng.* 1975, **17**, 1
56 Martinez, G. D. V., Ogawa, T., Shinmyo, A. and Enatsu, T. *Hakko Kogaku Zasshi* 1974, **52**, 378
57 Montenecourt, B. S. and Eveleigh, D. E. *Appl. Environ. Microbiol.* 1977, **34**, 777
58 Montenecourt, B. S., Eveliegh, D. E., Zlmund, G. K. and Parcells, J. *Biotechnol. Bioeng.* 1978, **20**, 297
59 Nisizawa, T., Suzuki, H. and Nisizawa, K. *J. Biochem.* 1971, **70**, 387
60 Nisizawa, T., Suzuki, H., Nakayama, M. and Nisizawa, K. *J. Biochem.* 1971, **70**, 375
61 Nisizawa, T., Suzuki, H. and Nisizawa, K. *J. Biochem.* 1972, **71**, 999
62 Nystrom, J. M. and DiLuca, P. H. in Reference 3, 1977, 293
63 Ohki, R. *J. Bacteriol.* 1975, **123**, 815
64 Palmer, J. K. *Appl. Polym. Symp.* 1975, **28**, 237
65 Peitersen, N. *Biotechnol. Bioeng.* 1975, **17**, 1291
66 Peitersen, N. *Biotechnol. Bioeng.* 1975, **17**, 361
67 Peitersen, N. in *Bioconversion of Cellulosic Substances into Energy, Chemicals and Microbial Proteins* (Ghose, T. K., ed.) Indian Institute of Technology Delhi, India, 1977, p. 281
68 Peitersen, N. *Biotechnol. Bioeng.* 1977, **19**, 337
69 Romanelli, R. A., Houston, C. W. and Barnet, S. M. *Appl. Microbiol.* 1975, **30**, 276
70 Ryu, D., Andreotti, R., Mandels, M., Gallo, B. and Reese, E. T. *Biotechnol. Bioeng.* in press
71 Sahai, V. and Ghose, T. K. in *Bioconversion of Cellulosic Substances into Energy, Chemicals and Microbial Proteins* (Ghose, T. K., ed.) Indian Institute of Technology Delhi, India, 1977, p. 269
72 Sattarova, R. K., Tashpulatov, Zh. and Safiyazov, Zh. *Mikrobiologiya* 1972, **41**, 413
73 Scherbakov, M. A. *Izv. Akad. Nauk. Mold SSR Ser. Biol. Khim. Nauk.* 1974, **1**, 45
74 Selby, K. in *Cellulases and Their Applications, Advances in Chem. Series 95* (Hajny, G. J. and Reese, E. T., eds) American Chemical Society, Washington, D.C., 1969, p. 34
75 Sevoyan, G. G., Sarukhanyan, F. G., Stephanyan, M. L., Akhinyan, R. M., Karimyan, R. S. and Petrosyan, L. G. *Biol. Zh. Arm.* 1976, **29**, 57
76 Shanghai Institute of Plant Physiology *Wei Sheng Wu Hsueh Pao* 1978, **18**, 27
77 Shin, S. B., Kitagawa, Y., Suga, K. and Ichigawa, K. *J Ferment. Technol.* 1978, **56**, 396
78 Simmons, E. G. *Abst. 2nd Int. Mycol. Cong.* 1977, 618
79 Skinner, W. A. and Tokuyama, F. US Pat. 4 081 328, (1978), Appl. 624 865, 6
80 Sternberg, D. and Dorval, S. *Biotechnol. Bioeng.* 1979, **21**, 181
81 Sternberg, D., Vijayakumar, P. and Reese, E. T. *Can. J. Microbiol.* 1977, **23**, 139
82 Sternberg, D. *Biotechnol. Bioeng.* 1976, **18**, 1751
83 Sternberg, D. in *Enzymatic Conversion of Cellulosic Materials: Technology and Application, Biotechnol. Bioeng. Symp. No. 6* (Gaden, Jr, E. L., Mandels, M. H., Reese, E. T. and Spano, L. A., eds) John Wiley and Sons, New York, 1976, p. 35
84 Sternberg, D. *Appl. Environ. Microbiol.* 1976, **31**, 648
85 Stutzenberger, F. J. *Appl. Microbiol.* 1971, **22**, 147
86 Toyama, N. and Ogawa, K. in *Bioconversion of Cellulosic Substances into Energy, Chemicals and Microbial Proteins* (Ghose, T. K., ed.) Indian Institute of Technology Delhi, India, 1977, p. 305
87 Ullmann, A., Tillier, F. and Menod, J. *Proc. Natl Acad. Sci. USA* 1976, **73**, 3476
88 Vilela, L. C., Torillo, A. R., DeOcampo, A. T. and Del Rosario, E. J. *Agric. Biol. Chem.* 1977, **41**, 235
89 Von Stockar, U., Der Yang, R. and Wilke, C. R. *Biotechnol. Bioeng.* 1977, **19**, 445
90 Wang, L. H. and Yuan-Chi, S. *Mem. Col. Agric. Natl. Taiwan Univ.* 1968, **9**, 1
91 Wood, T. M. in *Cellulose as a Chemical and Energy Resource, Biotechnol. Bioeng. Symp. No. 5* (Wilke, C. R., ed.) John Wiley and Sons, New York, 1975, p. 111
92 Wood, T. M. and Phillips, D. R. *Nature (London)* 1969, **222**, 986
93 Wood, T. M. and McCrae, S. in *Bioconversion of Cellulosic Substances into Energy, Chemicals and Microbial Proteins* (Ghose, T. K., ed.) Indian Institute of Technology Delhi, India, 1977
94 Zeltins, R. and Edzins, I. *Mikrobiol. Prep.* 1976, **51**
95 Tassinari, T. and Macy, C. unpublished results
96 Sternberg, D. and Mandels, G. *J. Bacteriol.* 1979, **139**, 741
97 Loewenberg, J. R. and Chapman, C. M. *Arch. Microbiol.* 1977, **113**, 61
98 Berg, B. and Pettersson, G. *J. Appl. Bacteriol.* 1977, **42**, 65
99 Mandels, M., Sternberg, D. and Andreotti, R. in *Bioconversion of Cellulosic Substances into Energy, Chemicals and Microbial Proteins* (Ghose, T. K., ed.) Indian Institute of Technology Delhi, India, 1977, p. 81
100 Academia Sinica *Wei Sheng Wu Hsueh Pao* 1977, **17**, 137
101 Andren, R. K., Mandels, M. H. and Medeiros, J. E. *Appl. Polym. Symp.* 1975, **28**, 205
102 Andren, R. K., Erickson, R. J. and Medeiros, J. E. in *Enzymatic Conversion of Cellulosic Materials: Technology and Applications, Biotechnol. Bioeng. Symp. No. 6* (Gaden, Jr, E. L., Mandels, M. H., Reese, E. T. and Spano, L. A., eds) John Wiley and Sons, New York, 1976, p. 177
103 Andren, R. K., Mandels, M. and Medeiros, J. E. *Process Biochem.* 1976, **11**, 2
104 Andren, R. K. in *Bioconversion of Cellulosic Substances into Energy, Chemicals and Microbial Proteins* (Ghose, T. K., ed.) Indian Institute of Technology Delhi, India, 1977, p. 397
105 Autrey, K. M., McCaskey, T. A. and Little, J. A. *J. Dairy Sci.* 1974, **57**, 132
106 Autrey, K. M., McCaskey, T. A. and Little, J. A. *J. Dairy Sci.* 1975, **58**, 67
107 Brandt, D., Hontz, L. and Mandels, M. *AIChE Symp.* 1973, **69**, 127
108 Cowling, E. B. in *Cellulose as a Chemical and Energy Resource, Biotechnol. Bioeng. Symp. No. 5* (Wilke, C. R., ed.) John Wiley and Sons, New York, 1975, p. 163
109 Crawford, D. L. and Crawford, R. L. *Appl. Environ. Microbiol.* 1976, **31**, 714
110 Deanin, R. D. *Appl. Polym. Symp.* 1975, **28**, 71
111 Dennis, C. *J. Gen. Microbiol.* 1972, **71**, 409
112 Elder, A. L. in *Enzymatic Conversion of Cellulosic Materials: Technology and Applications, Biotechnol. Bioeng. Symp. No. 6* (Gaden, Jr, E. L., Mandels, M. H., Reese, E. T. and Spano, L. A., eds) John Wiley and Sons, New York, 1976, p. 275
113 Eriksson, K. E., Pettersson, B. and Westermark, U. *Biol. Transform. Wood Microorg., Proc. Sess.* 1975, 143
114 Ghose, T. K. and Kostick, J. A. in *Cellulases and Their Applications, Advances in Chem. Series 95* (Hajny, G. J. and Reese, E. T., eds) American Chemical Society, Washington, D. C., 1969, p. 415
115 Ghose, T. K. and Kostick, J. A. *Biotechnol. Bioeng.* 1970, **12**, 921
116 Ghose, T. K. US Pat. 3 642 580, (1972), 5
117 Gupta, J. K., Gupta, Y. P. and Das, N. B. *Agric. Biol. Chem.* 1973, **37**, 2662
118 Guzhova, E. P., Burdenko, L. G. and Loginova, L. G. *Prikl. Biokhim. Mikrobiol.* 1976, **12**, 587
119 Howell, J. A. and Stuck, J. D. *Biotechnol. Bioeng.* 1975, **17**, 873
120 Howell, J. A. and Mangat, M. *Biotechnol. Bioeng.* 1978, **20**, 847
121 Huang, A. A. in *Cellulose as a Chemical and Energy Resource, Biotechnol. Bioeng. Symp. No. 5* (Wilke, C. R., ed.) John Wiley and Sons, New York, 1975, p. 245
122 Huang, A. A. *Biotechnol. Bioeng.* 1975, **17**, 1421
123 Kaneshiro, T., Kelson, B. F. and Sloneker, J. H. *Appl. Microbiol.* 1975, **30**, 876
124 Katz, M. and Reese, E. T. *Appl. Microbiol.* 1968, **16**, 419
125 Kirk, T. K. in *Cellulose as a Chemical and Energy Resource, Biotechnol. Bioeng. Symp. No. 5* (Wilke, C. R., ed.) John Wiley and Sons, New York, 1975, p. 139
126 Korculanin, Antea., Biocic, Ivka. and Juric, Zlata. *Prehrambeno-Tehnol. Rev.* 1977, **15**, 77
127 Krinstad, K. P. and Cheng, C. W. *Tappi J.* 1969, **52**, 2382
128 Lee, S. E., Arminger, W. B., Watteeuw, C. M. and Humphrey, A. E. *Biotechnol. Bioeng.* 1978, **20**, 141
129 Mandels, M. and Reese, E. T. *Dev. Ind. Microbiol.* 1964, **5**, 5
130 Mandels, M., Hontz, L. and Nystrom, J. *Biotechnol. Bioeng.* 1974, **16**, 1471
131 Mandels, M., Kostick, J. and Parizek, R. *J. Polym. Sci. (C)* 1971, **36**, 445
132 Mandels, M., Dorval, S. and Medeiros, J. in *Proceedings of 2nd Annual Symposium on Fuels from Biomass* (Shuster, W. W., ed.) John Wiley and Sons, New York, 1978, p. 627
133 McQueen, R. E. and Van Soest, P. J. *J. Dairy Sci.* 1973, **56**, 680

134 Menzies, J. W., Ger. Offen. 1 900 796, 11 Sept. 1969, US Appl. Jan 9, 1968, Nov 25, 1968, 22
135 Meyers, S. P. *Off. Naval Res., Arlington, Va., Rpt.* 1976, **18**, 15
136 Millett, M. A., Baker, A. J. and Satter, L. D. in *Enzymatic Conversion of Cellulosic Materials: Technology and Applications, Biotechnol. Bioeng. Symp. No. 6* (Gaden, Jr, E. L., Mandels, M. H., Reese, E. T. and Spano, L. A., eds) John Wiley and Sons, New York, 1976, p. 125
137 Morrison, L. M. *Biochem. Soc. Trans.* 1975, **3**, 992
138 Mukhopadhyay, S. M., Ghosh, P. and Potty, V. H. *J. Food Sci. Technol.* 1975, **12**, 120
139 Park, D. *Trans. Br. Mycol. Soc.* 1976, **66**, 65
140 Peitersen, N., Medeiros, J. and Mandels, M. *Biotechnol. Bioeng.* 1977, **19**, 1091
141 Pettersson, L. G., Axiö-Fredriksson, U. and Berghem, L. E. R. *Ferment. Technol. Today* 1972, 727
142 Pettersson, L. G. in *Symposium on Enzymatic Hydrolysis of Cellulose* (Bailey, M., Enari, T. M. and Linko, M., eds) The Finnish National Fund for Research and Development (SITRA), Helsinki, 1975, p. 255
143 Reese, E. T., Siu, R. G. H. and Levinson, H. S. *J. Bacteriol.* 1950, **59**, 485
144 Reese, E. T. in *Recent Advances in Phytochemistry – The Structure Biosynthesis and Degradation of Wood* (Loewus, F. and Runeckles, V. C., eds) Plenum Press, New York, 1977, 311
145 Riaz, Mohammad., Wilke, Charles R., Yamanaka, Yoshio., Carroad and Paul A. *Proc. Symp. Res. Appl. Natl. Needs* 1977, **2**, 108
146 Rinaudo, M., Barnoud, F. and Merle, J. P. in *Polym. Sci. (C) Polym. Symp.* Sixth Cellulose Conf. Proc. (Marschessault, R. H., ed.) John Wiley and Sons, New York, 1969, 197
147 Savarese, John J. and Young, Steven D. *Biotechnol. Bioeng.* 1978, **20**, 1291
148 Schauer, H., Annemueller, W., Heidrick, S. and Huber, J. Ger. Offen. 1 950 729 (Cl. C12 d) Jun 18, 1970, Germany (East) Appl. Oct 24, 1968, 10
149 Seely, D. B. in *Enzymatic Conversion of Cellulosic Materials: Technology and Applications, Biotechnol. Bioeng. Symp. No. 6* (Gaden, Jr, E. L., Mandels, M. H., Reese, E. T. and Spano, L. A., eds) John Wiley and Sons, New York, 1976, p. 285
150 Sinner, M., Parameswaran, N., Yamazaki, N., Liese, W. and Dietrichs, H. H. *Appl. Polym. Symp.* 1976, **28**, 993
151 Spano, L. A., Medeiros, J. and Mandels, M. *J. Wash. Acad. Sci.* 1976, **66**, 279
152 Spano, L. *J. Coatings Technol.* 1978, **50**, 71
153 Srinivasan, V. R., Fleenor, M. B. and Murray, E. *Dev. Ind. Microbiol.* 1972, **13**, 47
154 Su, T. M. and Paulavicius, I. *Appl. Polym. Symp.* 1975, **28**, 221
155 Tassinari, T. and Macy, C. *Biotechnol. Bioeng.* 1977, **19**, 1321
156 Terry, R. A., Tilley, J. M. A. and Outen, G. E. *J. Sci. Food Agric.* 1969, **20**, 317
157 Toyama, N. and Ogawa, K. *Ferment. Technol. Today* 1972, 743
158 Toyama, N. and Ogawa, K. in *Cellulose as a Chemical and Energy Resource, Biotechnol. Bioeng. Symp. No. 5* (Wilke, C. R., ed.) John Wiley and Sons, New York, 1975, p. 225
159 Toyama, N. in *Enzymatic Conversion of Cellulosic Materials: Technology and Applications, Biotechnol. Bioeng. Symp. No. 6* (Gaden, Jr, E. L., Mandels, M. H., Reese, E. T. and Spano, L. A., eds) John Wiley and Sons, New York, 1976, p. 207
160 Wilke, C. R. and Mitra, G. US Pat. 3 972 775, June 1974, 6
161 Wilke, Charles R., Stockar, Urs. V. and Yang, Ren. Der. *AIChE Symp. Ser.* 1976, **72** (158), 104
162 Wood, T. M. and McCrae, S. I. *Adv. Chem. Ser.* in press
163 Wood, T. M. and McCrae, S. I. *Biochem. J.* 1978, **171**, 61
164 Yamanaka, Y. and Wilke, C. R. *Effect of β-glucosidase on the Enzymic Hydrolysis of Cellulose* Lawrence Berkeley Lab., University of California, Berkeley, Calif. 1975, LBL-4413, 214
165 Zhbankov, R. G., Marupov, R. M., Shishko, A. M., Skrigan, A. L., Volkovich, S. M., Ivanova, I. V. and Korolik, E. V. *Izv. Akad. Nauk Tadzh. SSR. Otd. Fiz-Mat. Geol-Khim. Nauk* 1976, **3**, 66
166 Sasaki, T., Tanaka, T., Nanbu, N., Sato, Y. and Kainuma, K. *Biotechnol. Bioeng.* 1979, **21**, 1031
167 Mandels, M. and Reese, E. T. *Ann. Rev. Phytopathol.* 1965, **3**, 85
168 Berghem, L. E. R. and Pettersson, L. G. *Eur. J. Biochem.* 1974, **46**, 295
169 Emert, G. H., Gum, Jr, E. K., Lang, J. A., Liu, T. H. and Brown, Jr, R. D. *Adv. Chem. Ser.* 1974, **136**, 79
170 Gum, Jr, E. K. and Brown, Jr, R. D. *Biochem. Biophys. Acta* 1976, **446**, 371
171 Gum, Jr, E. K. and Brown, Jr, R. D. *Biochim. Biophys. Acta* 1977, **492**, 225
172 Shoemaker, S. P. and Brown, Jr, R. D. *Biochim. Biophys. Acta* 1978, **523**, 133
173 Shoemaker, S. P. and Brown, Jr, R. D. *Biochim. Biophys. Acta* 1978, **523**, 147
174 Berghem, L. E. R. and Pettersson, L. G. *Eur. J. Biochem.* 1973, **37**, 21
175 Berghem, L. E. R. and Pettersson, L. G. *Eur. J. Biochem.* 1975, **53**, 55
176 Halliwell, G. and Griffin, M. *Biochem. J.* 1973, **135**, 587
177 Fagerstam, L. G. and Pettersson, L. G. *FEBS Lett.* 1979, **98**, 363
178 Li, L. H., Flora, R. M. and King, K. W. *Arch. Biochem. Biophys.* 1965, **111**, 439
179 Nisizawa, K. *J. Ferment. Technol.* 1973, **51**, 267
180 Mandels, M. *Proc. 3rd Annu. Biomass Energy Syst. Conf.* 1979, p. 285
181 Reese, E. T. and Mandels, M. *Biotechnol. Bioeng.* 1980, **22**, 323
182 Bomar, M. T. and Schmid, S. *Aliment.* 1975, **14**, 62
183 Compere, A. L. and Griffiths, W. L. *Fermentation of Waste Materials to Produce Industrial Intermediates* Oak Ridge, Natl. Lab., Tenn., 1975, **18**, 17
184 Cysewski, G. R. and Wilke, C. R. *Biotechnol. Bioeng.* 1976, **18**, 1297
185 Dambrine, F., Giorgi, J. C. and Frouin, J. *Société Fives Lille-Cail, Fr.* 1 539 107, Cl. C07d, D21c, Sept 13, 1968, Appl. Aug 1, 1967, 3
186 Davis, H. G. in *Cellulose as a Chemical and Energy Resource, Biotechnol. Bioeng. Symp. No. 5* (Wilke, C. R., ed.) John Wiley and Sons, New York, 1975, p. 339
187 Edwards, V. H. *ibid*, p. 321
188 Eriksson, K. E. and Larsson, K. *Biotechnol. Bioeng.* 1975, **17**, 327
189 Eriksson, K. E. *Appl. Polym. Symp.* 1975, **28**, 197
190 Ferugson, S. R. US Pat. 3 875 317 (Cl 426-373; A23k) Apr 1, 1975, 181, 319, Sept 17, 1971, 5
191 Finn, R. K. in *Cellulose as a Chemical and Energy Resource, Biotechnol. Bioeng. Symp. No. 5* (Wilke, C. R., ed.) John Wiley and Sons, New York, 1975, p 279
192 Miller, D. L. *ibid*, p. 345
193 Fukomoto, J., Tsujisaka, Y., Okada, S. and Yamamoto, T. in *Proc. Symp. on Conversion and Manufacture of Foodstuffs by Microorganisms, Kyoto, Japan* 1972
194 Funk, H. F. *Appl. Polym. Symp.* 1975, **28**, 145
195 Gauss, W., Suzuki, S. and Takagi, M. 'Alcohol from Cellulose', Ger. Offen. 2 541 960 Apr 8, 1976, Jpn. Appl. 74 109 335, Sept 20, 1974
196 Goldstein, I. S. in *Enzymatic Conversion of Cellulosic Materials: Technology and Applications, Biotechnol. Bioeng. Symp. No. 6* (Gaden, Jr, E. L., Mandels, M. H., Reese, E. T. and Spano, L. A., eds) John Wiley and Sons, New York, 1976, p. 293
197 Hearon, W. M., Lo, C. F. and Witte, J. F. *Appl. Polym. Symp.* 1975, **28**, 77
198 Jaffe, G. M., Szkrbalo, W., Weinert, H. US Pat. 3 784 408, Cl 127/37; CL3K, Jan 8, 1974, Appl. 863 558, Oct 29, 1969, 5
199 Linko, M. *Kem.-Kemi* 1975, **2**, 602
200 Meyers, S. E. *Proc. Pa. Chec. '77 AIChE* Denver, Colorado, 1977, 781
201 Moo-Youn, M. *Can. J. Chem. Eng.* 1975, **53**, 113
202 McLoughlin, A. J. *Irish Forestry* 1972, **29**, 15
203 Nagy, G., Balint, K. E. and Sos, A. *Elelmez. Ipar.* 1975, **29**, 41
204 Peking Institute of Microbiology *Wei Sheng Wu Hsueh Pao.* 1977, **17**, 231
205 Rogers, C. J. *J. Wash. Acad. Sci.* 1976, **66**, 271
206 Savage, J. *Diss. Abs. Int. (B)* 1975, **35**, 5015
207 Stefoglo, E. F., Ermakova, A., Vasyunina, N. A. and Klabunovskii, E. I. *Khim. Pram. (Moscow)* 1973, **49**, 576
208 Takagi, M., Abe, S., Suzuki, S., Emert, G. H. and Yata, N. in *Bioconversion of Cellulosic Substances into Energy, Chemicals and Microbial Proteins* (Ghose, T. K., ed.) Indian Institute of Technology Delhi, India, 1977, p. 551
209 Wang, D. I. C., Cooney, C. L., Wang, Sy-dar, Gordon J. and Wang, G. Y. in ref 7. p. 537

210 Schewale, J. G. and Sadana, J. C. *Can. J. Microbiol.* 1979, **25**, 773

211 Bastawde, K. B., Vasonti, V., Deshponde, A. V., Joglekar, B. C., Lakshrni, K., Chittra, M., Phansalkar, S. B., Mala, R., Seeta, R., Srinivasan, M. C. and Jagannathan, V. in *Biocon-version of Cellulosic Substances into Energy, Chemicals and Microbial Proteins* (Ghose, T. K., ed.) Indian Institute of Technology Delhi, India, 1977, p. 387

212 Ferchak, T. D., Hagerdal, B. and Pye, K. E. *Biotechnol. Bioeng.* in press